Leukemia Research 36 (2012) 921–930

Leukemia Research

journal homepage: www.elsevier.com/locate/leukres

Azacitidine differentially affects CD4^{pos} T-cell polarization *in vitro* and *in vivo* in high risk myelodysplastic syndromes

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ARTICLE INFO

Article history:
Received 27 November 2011
Received in revised form 26 March 2012
Accepted 26 March 2012
Available online 14 April 2012

Keywords:
Azacitidine
Cytokines
Regulatory T-cells
IL-17
CD4 T-helper cells
MDS

ABSTRACT

CD4^{pos} T-cell subsets play a role in myelodysplastic syndromes (MDS) pathogenesis and may be affected upon 5-azacitidine (Aza) treatment. Aza enhanced human T_H1 frequencies *in vitro* but not *in vivo*. The proportion of functional FoxP3^{pos} regulatory T cells (Treg) was enhanced by Aza *in vitro* ($p < 0.0002$), and a modest, temporary increase was observed *in vivo* ($p = 0.08$). The overall number of T_H17 was reduced both *in vitro* ($p < 0.03$) and *in vivo* ($p < 0.006$), indicating that Aza directly affects CD4^{pos} polarization during activation *in vitro*. Upon *in vivo* treatment in high risk MDS patients, particularly the T_H17-Treg axis is affected.

1. Introduction

Demethylating agents such as the DNA methyltransferase (DNMT) inhibitor 5-azacitidine (Aza) are increasingly used for the treatment of myelodysplastic syndromes (MDS), acute myeloid leukemia (AML) and multiple myeloma (MM) [1–4]. Treatment with Aza was shown to increase overall survival in high risk MDS patients as compared to conventional care [5]. The mechanisms behind the therapeutic effects of Aza are not yet clear, differentiation induction, direct cytotoxic effects on dysplastic clones as well as enhanced expression of tumor-associated antigens (TAA), which are targets for the immune response are likely to contribute to the outcome of treatment [6–8].

Immune editing is recognized as being important in the pathogenesis of MDS [9]. Over-production of pro-inflammatory cytokines such as TNFα and to a lesser extend IFNγ appears to accelerate apoptosis of haematopoietic progenitors in the bone marrow and thus contribute to the disturbed haematopoiesis in low-risk MDS [10,11]. The role of IL-17 is less clear. Increased frequencies of IL-17 producing CD4^{pos} T_H17 cells have been observed in peripheral blood of low risk patients suggesting a role for increased autoimmunity in low risk MDS and a possible role in an effective immune response against the dysplastic clone [12]. On the other hand in the bone marrow of early MDS cases T_H17 frequencies were reduced and produced less IL-21 and IL-22, as compared to late MDS cases [13]. In addition to abnormal production of inflammatory cytokines immune evasion characterized by increased numbers of FoxP3^{pos} Treg may be related to the outgrowth of (transformed) blasts in high risk MDS cases [13,14].

Next to direct effects of Aza on dysplastic and/or tumor cells, modulation of lymphocyte effector functions are likely to occur, as both cytokine production and FoxP3 expression by T-cells have been shown to be epigenetically regulated *in vitro* [15,16]. The promoter of FoxP3, a key gene for the function of CD25^{hi} regulatory T-cells (Treg), is methylated in conventional CD4^{pos}CD25^{neg} T-cells [17]. It has been demonstrated previously that treatment with DNMT inhibitors induces conversion of human CD4^{pos}CD25^{neg} conventional T-cells into CD25^{pos}FoxP3^{pos} cells with regulatory function [15]. Recent studies in transplantation models suggest that Aza may be an effective treatment for graft *versus* host disease particular due to its combined effects on TAA expression and FoxP3 expression [18,19].

However, effects of Aza treatment on (inflammatory) cytokine production are less clear; conflicting results regarding IFNγ production upon treatment with demethylating agents have been reported [16,18,20,21] while epigenetic control of TNFα and IL-17 production, both of which are related to the increased bone marrow apoptosis in low risk MDS, have been less extensively studied

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0145-2126
http://dx.doi.org/10.1016/j.leukres.2012.03.026

Table 1
Patient's characteristics.

Ptnt#	Age	FAB	WHO 2008	Response	T-cell monitoring	Time in FU
1	69	RAEB-t	AML with multilineage dysplasia	Responder	$T=0$, 15 days, 3 cycles	9 cycles, pause due to toxicity
2	74	RAEB	RAEB-2	Stable	$T=0$, 15 days, 3 cycles	12 cycles, ongoing
3	67	RAEB	RAEB-2	Progression	$T=0$, 15 days	6 cycles, progressive disease
4	71	RAEB	RAEB-2	Responder	$T=0$, 15 days	9 cycles, ongoing
5[a]	74	RAEB	RAEB-2	Progression	$T=0$, 15 days	Died of disease after 2 cycles
6	72	RAEB-t	AML with multilineage dysplasia	Progression	$T=0$, 15 days, 3 cycles	Died of disease after 3 cycles
7	66	CMML	CMML-1	Stable	$T=0$, 15 days, 3 cycles	3 cycles, pause due to toxicity
8	74	RAEB	RAEB-2	Progression	$T=0$, 15 days, 3 cycles	3 cycles, progressive disease
9	72	CMML	CMML-2	Progression	$T=0$, 15 days, 3 cycles	3 cycles, progressive disease

[a] No cytokines measured due to a technical error.

[10,12,21]. Furthermore, *in vivo* effects of Aza on Treg development and cytokine production in humans have not yet been reported.

We therefore set out to investigate the effects of Aza treatment on $CD4^{pos}$ T-cell subset frequencies *in vivo*, in high-risk MDS patients. In addition the effect of Aza treatment on the conversion of conventional $CD4^{pos}$ T-cells into FoxP3 expressing Treg, as well as on the frequency of IFNγ, TNFα and IL-17 producing T-cells was analyzed in healthy donors *in vitro*.

2. Materials and methods

2.1. Study design and patients

Patients with intermediate-2 and high risk MDS ($n=9$, IPSS score ≥ 1.5), CMML and AML with 20–30% blasts and multilineage dysplasia were included in this study. The study received local institutional review board approval. Patients received s.c. injections of 75 mg/m^2/day Aza on the first 7 days of a 28 day cycle. After informed consent, blood was drawn from 3 patients at the start of Aza treatment and at day 15 of cycle 1. Of an additional 6 patients blood was drawn after 3 completed cycles as well. Table 1 shows the characteristics of the patients.

2.2. Isolation of PBMC and T-cell subsets

Peripheral blood was acquired after informed consent from healthy blood donors and MDS patients. PBMC were isolated within 24 h of blood sampling using ficoll density-gradient centrifugation (Ficoll-Paque, Amersham Pharmacia Biotech, Uppsala, Sweden). For the *in vitro* assays, $CD4^{pos}$ T-cells were subsequently isolated by MACS sorting using the CD4 positive isolation kit from Miltenyi Biotec (Bergisch Gladbach, Germany). $CD4^{pos}$ cells were subsequently stained with CD25-PE (BD Biosciences, San Jose, CA, USA) and sorted into $CD25^{neg}$ and $CD25^{dim}$ subsets by sorting using a FACSaria flow cytometer (BD Biosciences, San Jose, CA) with a purity of typically >95%.

2.3. In vitro stimulation of isolated $CD4^{pos}$ subsets

20×10^6/ml $CD4^{pos}$ T-cells (total, $CD25^{neg}$ or $CD25^{dim}$) were labeled with CFSE (Molecular probes, Eugene, OR; 3 μM in PBS) for 10 min at 37 °C followed by 2 min incubation at room temperature upon addition of an equal volume of fetal calf serum (FCS, Greiner Bio-one, Alphen aan de Rijn, The Netherlands). After washing the cells were resuspended in IMDM culture medium supplemented with 0.01 mM β-mercaptoethanol and 50 units/ml penicillin-streptomycin (all from Gibco, Paisley, UK) and 5% FCS (10×10^6/ml). CD3 (4 μg/ml; Sanquin Reagents, Amsterdam, The Netherlands) labeling was subsequently performed during 1 h on ice. After washing the cells were resuspended to 4×10^5/ml IMDM/10% FCS and 500 μl/well was plated out on goat-anti-mouse IgG coated (DakoCytomation, Glostrup, Denmark) 48 well plates. Aza (Sigma–Aldrich, Saint Louis, MO) was dissolved in acetic acid (VWR, Fontenay-sous-Bois, France; 50% (v/v) in H_2O) to a concentration of 20 mM and used at 10 nM–10 μM. Freshly dissolved and diluted Aza or an equal volume of vehicle was added on day 1, 2 and 3, on day 6 the cells were harvested and CFSE dilution was determined by flow cytometry and the proliferation index was calculated by ModFit software.

2.4. Treg and cytokine production analysis

Peripheral blood T-cell subset frequencies in MDS patients were analyzed using six-color flow cytometry. FITC, PE, PerCP, APC, PE-Cy7 or APC-H7 conjugated monoclonal antibodies directed against the following markers were applied: CD45, CD3, CD4, CD8, CD127, IL-4, IFNγ, (all BD Biosciences), FoxP3 (e-Bioscience, San Diego, CA, USA), IL-17A and TNFα (Biolegend, San Diego, CA, USA). Data obtained from a minimum of 20,000 $CD4^{pos}$ cells acquired by a FACS Canto II flow cytometer using FACSDiva software were analyzed using CellQuestPro software. Absolute lymphocyte subset numbers were calculated as follows; absolute number of $CD3^{pos}$ T-cells: lymphocyte count × (%CD3 in lymphocyte gate/100) and absolute number of $CD3^{pos}CD4^{pos}$ T-cells: absolute $CD3^{pos}$ T-cell count × (%$CD4^{pos}$ cells within $CD3^{pos}$ lymphocyte gate/100). Results are shown as percentage of $CD3^{pos}CD4^{pos}$ T-cells. Treg cells were identified as $CD3^{pos}CD4^{pos}CD127^{lo/neg}FoxP3^{pos}$ (example of gating procedure is shown in Supplementary Fig. 1A). Intracellular FoxP3 staining was performed according to the manufacturer's instructions. Cytokine producing cells were identified after 16 h stimulation with phorbol 12-myristate 13-acetate (PMA, 2.5 ng/ml) and ionomycin (1 μg/ml), 10 μg/ml Brefeldin A was added after 1 h (all from Sigma–Aldrich). After surface staining with CD45, CD3 and CD8, the cells were fixed (1% (v/v) paraformaldehyde, Sigma–Aldrich) and permeabilized (0.1% saponin, Merck, Darmstadt, Germany) and antibodies against IL-17A, TNFα and IL-4 and IFNγ were added. $CD4^{pos}$ T-cells were defined as $CD3^{pos}CD8^{neg}$ (example of gating procedure is shown in Supplementary Fig. 1B), as cell surface expression of CD4 is significantly reduced after PMA/ionomycin stimulation, this population therefore includes double negative γδT-cells.

The *in vitro* stimulated healthy donor derived $CD4^{pos}$ T-cells were analyzed similarly. However, Treg were defined as $CD25^{hi}FoxP3^{pos}$ using CD25-PE and FoxP3-APC antibodies. After 6 days culture the cells were harvested and Treg frequencies were directly determined. The remaining cells were stimulated with PMA/ionomycin as described above. Upon fixation and permeabilization cells were stained with antibodies against IL-17A and TNFα combined with antibodies against IFNγ or FoxP3. Data were obtained from a minimum of 20,000 $CD4^{pos}$ cells in the live gate using the FACS Canto and FACSDiva software and were subsequently analyzed using CellQuest software (BD Biosciences).

2.5. Real time quantitative reverse transcriptase PCR

Cells were harvested and snap-frozen on day 6. RNA was isolated and reverse transcribed into cDNA as described previously [22]. Transcripts were quantified by real-time quantitative polymerase chain reaction on a ABI/PRISM 7500 sequence detection system using pre-designed TaqMan GeneExpression Assays and reagents according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). Probes with the following Applied Biosystems assay identification numbers were used: TBX21, Hs00203436_m1 (Tbet); RORC variant 1 and 2, Hs01076112_m1 (RoRγT) and FOXP3, Hs00203958_m1. Data were normalized using the human HPRT1 Endogenous Control (4333768T, Applied Biosystems).

2.6. Treg assay

After 6 days of culture in the presence of vehicle or Aza healthy donor derived $CD4^{pos}$ T-cells were harvested and stained with CD25-PE and sorted into $CD25^{hi}$ and $CD25^{dim/neg}$ cells, in order to enrich for $FoxP3^{pos}$ cells in the $CD25^{hi}$ population. These sorted cells as well as untreated $CD4^{pos}CD25^{neg}$ cells were labeled with PKH26 (2 μl/ml; Sigma–Aldrich) in PBS (1×10^6 cells/ml) for 10 min at room temperature. An equal volume of FCS was added and after additional 2 min incubation the cells were washed and resuspended in IMDM/10%FCS. Autologous total $CD4^{pos}$ T-cells were labeled with CFSE and anti-CD3 as described above ("Tresp"), washed and resuspended in IMDM/10%FCS and plated out at 50,000 cells per well on a goat-anti-mouse coated flat-bottomed well plate. 1×10^5 or 50,000 PKH62 labeled untreated, vehicle or Aza cultured $CD25^{neg}$ or vehicle or Aza cultured $CD25^{hi}$ ("Treg") were added in duplicate wells. After 4 days of culture the cells were harvested and CFSE dilution of the Tresp was determined after gating out the $PKH26^{pos}$ Treg.

2.7. Statistical analysis

The two sided paired Student's *t*-test and ANOVA analyses were used to compare the data. In all cases the data had a normal distribution as determined by the Kolmogorov–Smirnov test. *p*-Values <0.05 were considered significant.

3. Results

3.1. Aza inhibits proliferation but induces activation of CD4^{pos} T-cells in vitro

First a dose response experiment examining T-cell proliferation and FoxP3 expression was performed to establish the optimal dose for Aza. In this experimental setting, CD4^{pos} T-cell proliferation induced by CD3 triggering was inhibited in a dose dependent manner (Fig. 1a). The overall proportion of CD25^{hi}FoxP3^{hi} was increased by Aza at a concentration of 1 μM (Figs. 1b, c and 4a). This dose was previously also shown to be non-toxic and to induce FoxP3 expression in human CD4^{pos}CD25^{neg} T-cells [18,19]. During culture, a FoxP3^{dim} and a FoxP3^{hi} population emerged in both the vehicle as well as the Aza cultures (Fig. 1c). The FoxP3^{hi} population also expresses relatively high levels of CD25, suggesting that these are Treg, while the FoxP3^{dim}CD25^{pos} are likely to be activated conventional T-cells (Tconv) [23]. The overall increase in CD25 expression is also suggestive for an increased activation state upon culture in the presence of Aza ($p < 0.002$; Fig. 1d).

3.2. In vivo Aza treatment does not affect relative or absolute CD3^{pos}CD4^{pos} T-cell numbers

The frequency of circulating CD4^{pos} T-cell subsets was investigated in patients with int-2 and high risk MDS, CMML and AML undergoing Aza treatment. Blood samples were obtained from 9 patients before the start of treatment, 15 days after the start of treatment and for 6 patients at the end of the third cycle as well (Table 1). No changes in absolute number of CD3^{pos} T-cells (mean of 1.9; 1.7; 1.5×10^9/l), CD3^{pos}CD4^{pos} T-cells (mean of 1.1; 1.0; 1.0×10^9/l), or in the proportion of CD3^{pos}CD4^{pos} T-cells relative to CD45^{hi} lymphocytes (mean of 59.7, 60.8, 64.5%) were observed during treatment (all before, 15 days after the start of treatment and after 3 cycles, respectively).

3.3. Aza affects in vivo and in vitro cytokine production

The capacity of both Aza-cultured healthy donor-derived CD4^{pos} T-cells (Fig. 2a, c and e) and of CD4^{pos} T-cells from Aza treated MDS patients (Fig. 2b, d and f) to produce cytokines upon PMA/ionomycin stimulation was investigated (representative FACS dotplots are shown in Supplementary Fig. 2). The number of IFNγ as well as TNFα producing cells was increased by Aza, again demonstrating that Aza enhances *in vitro* T-cell activation (Fig. 2a and c). However, *in vivo*, no significant change was observed in the proportion of IFNγ or TNFα producing cells in MDS patients after Aza treatment (Fig. 2b and d). IL-17 producing cells can be divided into IFNγ negative T_H17 and IFNγ co-producing T_H17-1 cells. It has been suggested that these cells have a differential origin and function and were therefore analyzed separately. No significant changes in the proportion of IL-17^{pos}IFNγ^{pos} T_H17-1 cells were observed (not shown), however the proportion of IL-17^{pos}IFNγ^{neg} T_H17 cells was significantly reduced by Aza both in the *in vitro* culture system as well as *in vivo* in Aza treated MDS patients (Fig. 2e and f), leading to an increased IL-17^{pos}IFNγ^{pos}/IL-17^{pos}IFNγ^{neg} ratio ($p < 0.04$ data not shown).

3.4. Aza differentially affects cytokine production by CD25^{neg} and CD25^{pos} cells in vitro

While the number of IFNγ producing CD4^{pos} T-cells was consistently up-regulated by Aza in all donors upon *in vitro* stimulation, for TNFα this was only the case in 7/10 donors. We hypothesized that Aza differentially affected the activation of resting CD4^{pos}CD25^{neg} and previously activated CD4^{pos}CD25^{dim} T-cells and that differences in activated T-cell frequencies among the different donors may have caused this variation in TNFα producing cells. We therefore sorted the CD4^{pos} T-cell into recently activated CD25^{dim} cells and resting CD25^{neg} cells and analyzed whether Aza has differential effects on resting *versus* recently activated CD4^{pos} T-cells. Co-staining with CD45RO and CD27 showed that effector memory (CD45RO^{pos}CD27^{neg}) and central memory (CD45RO^{pos}CD27^{pos}) CD4^{pos} T-cells are CD25^{dim} and that naïve CD45RO^{neg}CD27^{pos}CD4^{pos} T-cells are predominantly CD25^{neg} (Fig. 3a).

The number of IFNγ producing cells was up-regulated by Aza both among CD25^{neg} as well as CD25^{dim} cells in all donors (Fig. 3b), while TNFα production was only affected in the CD25^{dim} population, where it was up-regulated in all donors (Fig. 3c). In contrast, the proportion of IL-17 producing cells, although very low, was consistently increased under the influence of Aza in the CD25^{neg} population (Fig. 3d). These data suggest that differential epigenetic regulation by DNA methylation plays a role in IL-17 and TNFα production by recently activated CD25^{dim} and resting CD25^{neg} CD4^{pos} T-cells.

3.5. Aza affects in vitro and in vivo Treg development

In vitro Aza treatment significantly increased the conversion into CD25^{hi}FoxP3^{hi} cells from the total population of CD4^{pos} T-cells ($p < 0.0002$; Fig. 4a), and from isolated CD4^{pos}CD25^{neg} as well as CD4^{pos}CD25^{dim} cells ($p < 0.004$, not shown). CD25^{hi} cells ceased to proliferate when cultured in the presence of Aza, indicating that the increase in CD25^{hi}FoxP3^{hi} cells among proliferating CD4^{pos} T-cells in the presence of Aza is due to conversion of conventional CD25$^{neg/dim}$ cells, rather than due to proliferation of CD25^{hi}FoxP3^{pos} cells (not shown). *In vivo* there was a trend toward an increase in the proportion of FoxP3^{pos} Treg within the CD3^{pos}CD4^{pos} T-cell population on day 15 of the first cycle but the number of FoxP3^{pos} Treg was reduced again to levels before treatment at the end of the third cycle (Fig. 4b).

It is known from literature that particularly the balance of effector T-cells (Teff) and Treg determines whether there is immunity or immune suppression [24,25]. Furthermore, an increased T_H17:Treg ratio in low risk MDS as compared to high risk MDS has been correlated to increased bone marrow apoptosis [12]. We, therefore, analyzed the effect of Aza treatment on the T_H17:Treg ratio as well as on the IFNγ:Treg and TNFα:Treg ratio's. Due to the (initial) increase in Treg and the decline in IL-17 producing T-cells, the T_H17:Treg ratio is significantly reduced in the *in vitro* cultures ($p < 0.05$; Fig. 4c) as well as *in vivo* 15 days after the start of treatment ($p < 0.02$; Fig. 4d). In contrast to T_H17, the number of IFNγ and TNFα producing cells was increased *in vitro* by Aza, nevertheless, due to the strong concomitant increase in Treg numbers, the IFNγ:Treg and TNFα:Treg ratio's were also significantly reduced when the cells were treated with Aza ($p < 0.03$; not shown).

To study whether the *in vitro* change in cytokine production was due to a change in expression of transcription factors related to IFNγ and IL-17; T-bet and RORγT respectively, QRT-PCR was performed on CD25^{neg} cells on day 6. No changes in T-bet and RORγT expression were observed, while FoxP3 mRNA expression was enhanced with borderline significance (Fig. 4e). These data suggest that Aza affects the methylation status of the cytokine promoters, allowing greater access to these transcription factors rather than affecting cytokine expression through modulation of transcription factor expression.

3.6. Aza induced CD25^{hi}FoxP3^{pos} cells have regulatory function

In humans FoxP3 is not only expressed by Treg but is also up-regulated in activated T-cells. As CD25 expression as well as the

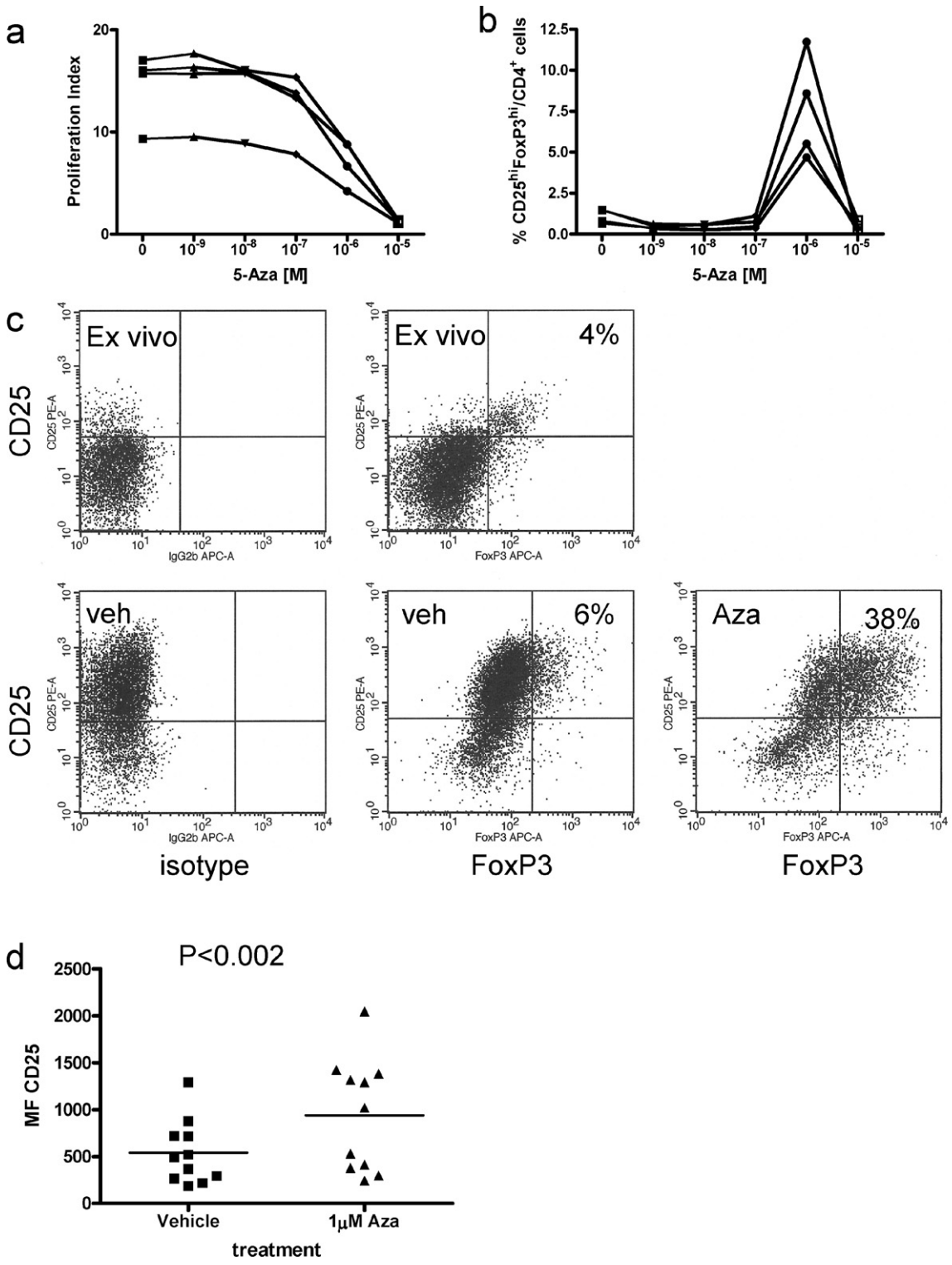

Fig. 1. *In vitro* effect of Aza on proliferation, FoxP3 and CD25 expression of $CD4^{pos}$ T-cells. Proliferation and activation of isolated $CD4^{pos}$ T-cells was induced by CD3 cross-linking. Vehicle or indicated concentrations of Aza were added at the start of the culture and on two consecutive days of culture, cells were analyzed on day 6. (a) Increasing concentrations of Aza inhibit $CD4^{pos}$ T-cell proliferation ($n = 4$). (b) FoxP3 expression is increased at 1 μM ($n = 4$). (c) Example of flowcytrometric analysis of CD25 and intracellular FoxP3 staining of pre-culture $CD4^{pos}$ T-cells (*ex vivo*), vehicle (veh) and Aza treated cells. Cells in the upper right quadrant represent cells with a Treg phenotype; the numbers represent the percentage of cells in the quadrant. (d) CD25 expression (MF = mean fluorescence intensity) is significantly up-regulated by Aza treatment ($n = 11$); $p < 0.002$; 2-sided paired Student's *t*-test.

proportion of IFNγ and TNFα producing cells is increased by Aza, the increase in FoxP3 expression may be a sign of T-cell activation rather than a true conversion of Tconv into Treg. To study this in further detail we performed FoxP3 double stainings with both IFNγ and TNFα (representative dotplots are shown in Supplementary Fig. 3). Indeed double positive cells could be detected but the proportion of FoxP3 single positive cells was significantly increased by Aza ($p < 0.001$, Fig. 5a), indicating that these may indeed be Treg rather than activated Tconv. To establish regulatory function, vehicle and Aza treated cells were sorted into $CD25^{hi}$ and $CD25^{dim/neg}$ cells as

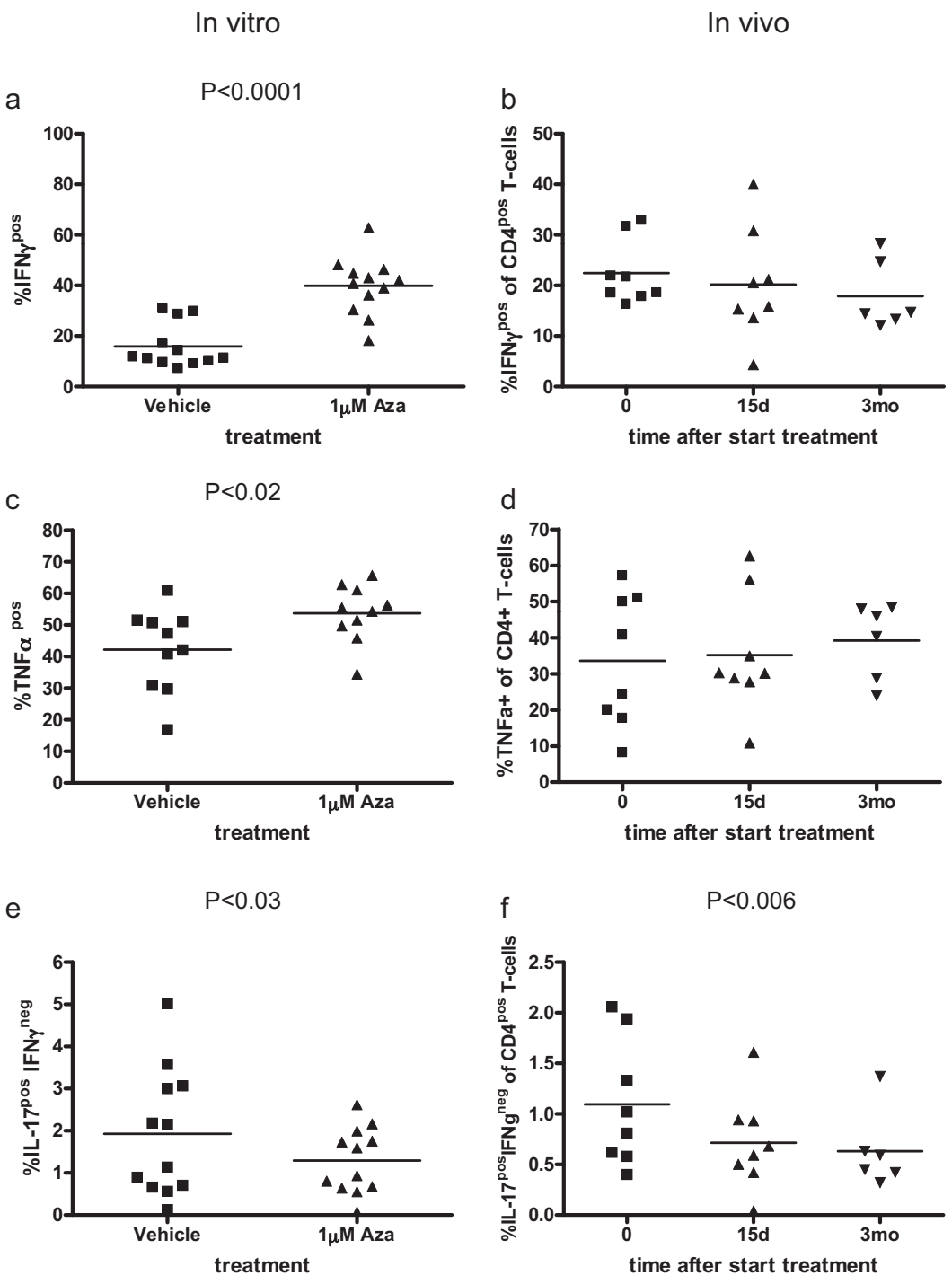

Fig. 2. *In vitro* and *in vivo* effect of Aza on IFNγ, TNFα and IL-17 production by $CD4^{pos}$ T-cells. (a, c, and e) Proliferation and activation of isolated $CD4^{pos}$ T-cells was induced by CD3 cross-linking. Vehicle or 1 μM of Aza was added at the start of the culture and on two consecutive days of culture, cells harvested on day 6 were analyzed for IFNγ ($n = 12$) TNFα ($n = 10$) and IL-17 ($n = 12$). (b, d, and f) *ex vivo* analysis of $CD3^{pos}CD4^{pos}$ cells of MDS patients before treatment ($n = 8$), on day 15 of the first cycle ($n = 8$) and after 3 cycles ($n = 6$). Cytokine production was analyzed after 16 h stimulation with PMA/ionomycin in the presence of Brefeldin A. (a and b) the proportion of IFNγ producing cells; (c and d) the proportion of TNFα producing cells; (e and f) the proportion of IL-$17^{pos}IFN\gamma^{neg}$ T_H17 cells. *p* values are from 2-sided paired Student's *t*-test (a, c, and e) and 2-sided ANOVA (f).

to enrich the $CD25^{hi}$ population for $FoxP3^{hi}$ cells (Fig. 5b). These four populations as well as uncultured $CD25^{neg}$ control cells were added to autologous $CD4^{pos}$ T-cells and their capacity to inhibit CD3-triggered proliferation was analyzed. Indeed $CD25^{hi}$ cells isolated from Aza treated cultures were highly capable of inhibiting $CD4^{pos}$ T-cell proliferation whereas the $CD25^{hi}$ isolated from vehicle treated cell inhibited proliferation to a much lesser extend (Fig. 5c). These data show that Aza indeed facilitates the conversion of Tconv into $FoxP3^{pos}$ Treg in this experimental setting.

4. Discussion

Immune evasion characterized by increased numbers of $FoxP3^{pos}$ Treg [14] as well as abnormal production of inflammatory

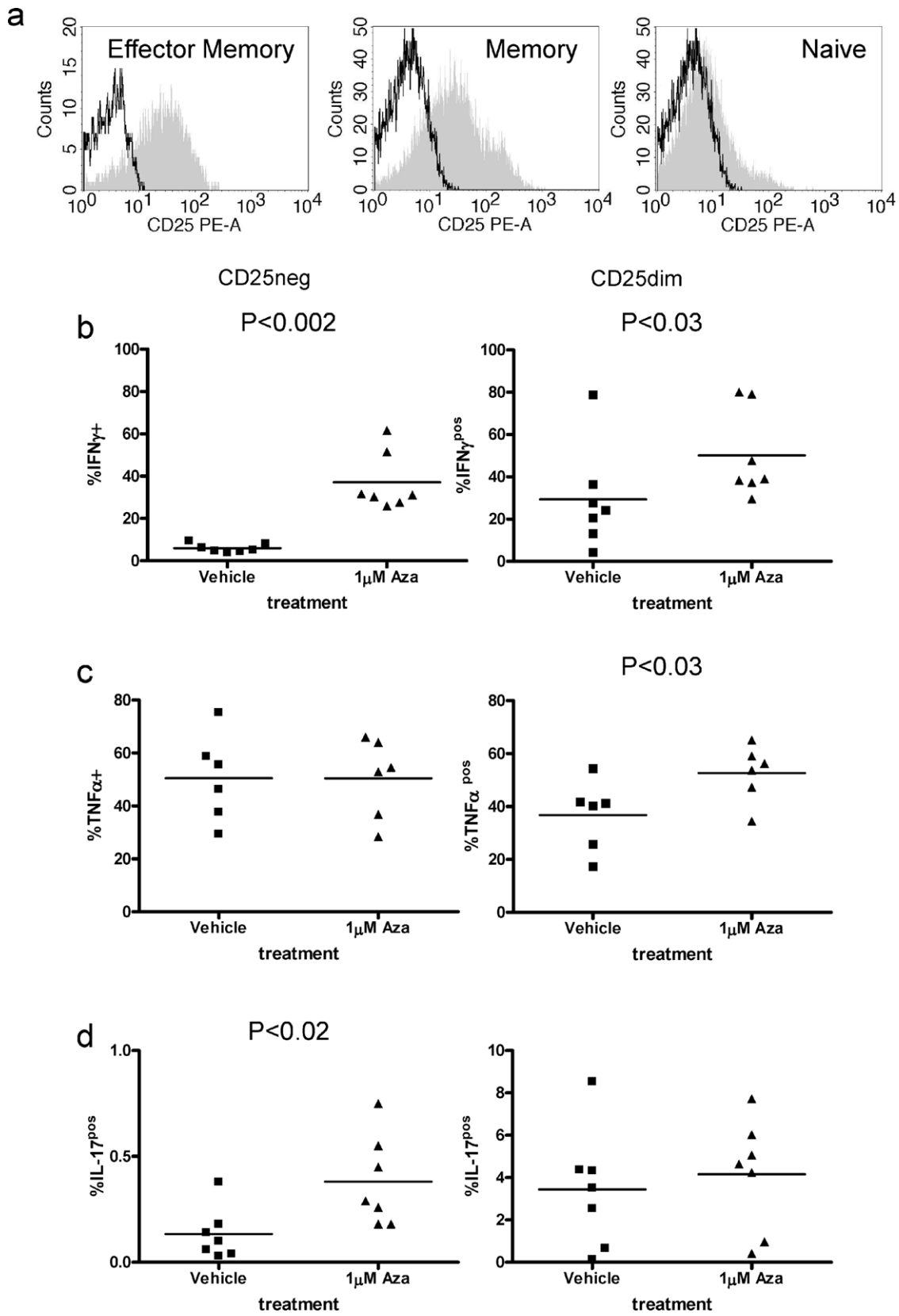

Fig. 3. *In vitro* effect of Aza on IFNγ, TNFα and IL-17 production by $CD4^{pos}CD25^{neg}$ and $CD4^{pos}CD25^{dim}$ T-cells. (a) $CD45RO^{pos}CD27^{neg}$ effector memory, $CD45RO^{pos}CD27^{pos}$ central memory and $CD45RO^{neg}CD27^{pos}$ naïve CD4+ T-cells were gated and the level of CD25 expression is shown. Open histograms: isotype control; closed gray histograms: CD25. (b–d) Proliferation and activation of isolated $CD4^{pos}CD25^{neg}$ and $CD4^{pos}CD25^{dim}$ T-cells was induced by CD3 cross-linking. Vehicle or 1 μM of Aza was added at the start of the culture and on two consecutive days of culture, cells were harvested on day 6. The frequency of (b) IFNγ ($n = 7$), (c) TNFα ($n = 6$) and (d) IL-17 ($n = 7$) producing cells after 16 h stimulation with PMA/ionomycin is shown. Left panels: results from $CD25^{neg}$ cultured cells; right panels: results from $CD25^{dim}$ cultured cells. *p*-Values are from 2-sided paired Student's *t*-tests.

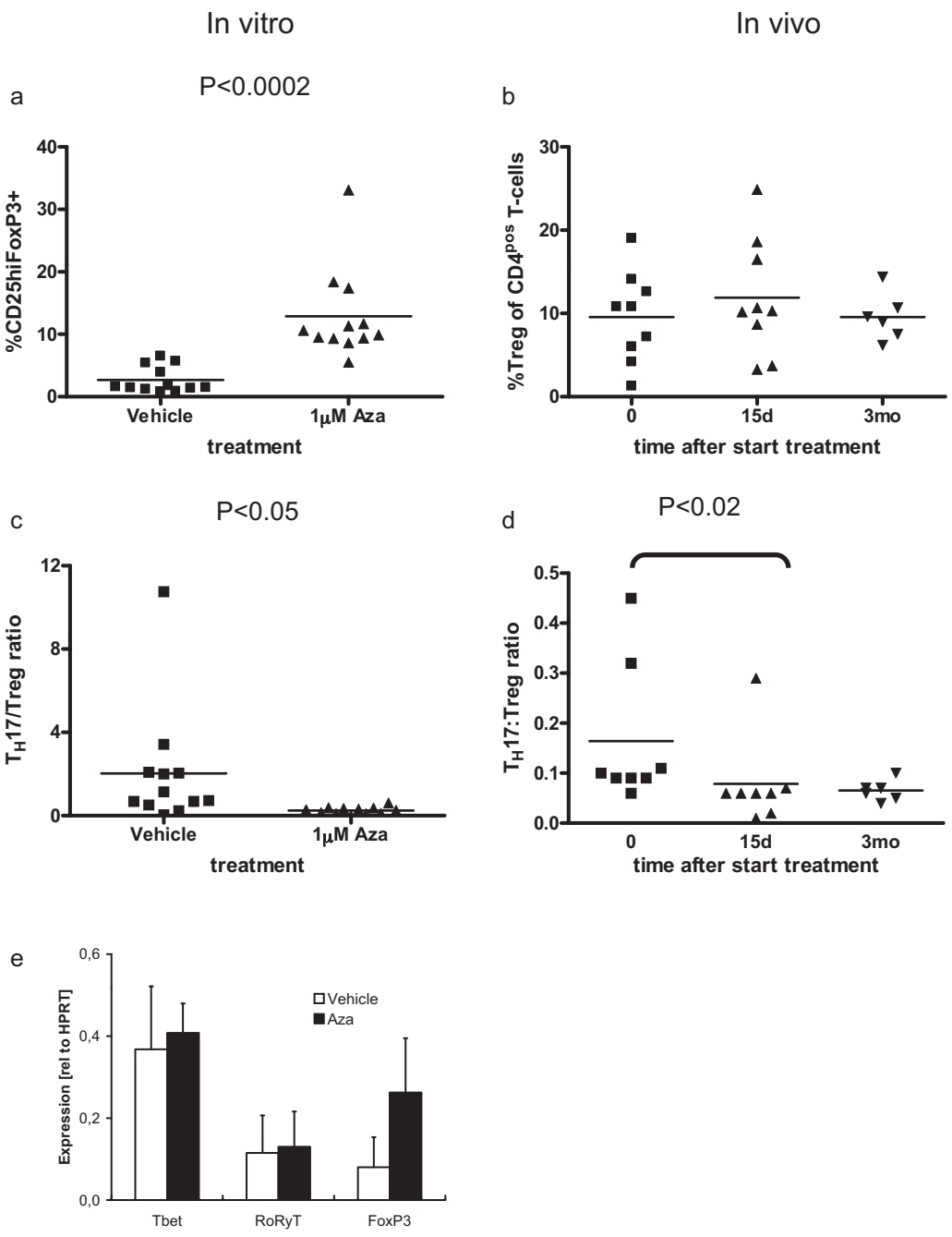

Fig. 4. *In vitro* and *in vivo* effect of Aza on the development of Treg. (a and c) Proliferation and activation of isolated $CD4^{pos}$ T-cells or (e) $CD4^{pos}CD25^{neg}$ was induced by CD3 cross-linking. Vehicle or 1 μM of Aza was added at the start of the culture and on two consecutive days of culture, cells were harvested on day 6. Treg were defined as (a) $CD25^{hi}FoxP3^{hi}$ in Aza cultured cells ($n = 12$) or (b) as $CD3^{pos}CD4^{pos}CD127^{lo/neg}FoxP3^{pos}$ in PBMC of Aza treated MDS patients before treatment ($n = 9$), on day 15 of the first cycle ($n = 9$) and after 3 cycles ($n = 6$). T_H17:Treg ratio among (c) *in vitro* cultured cells ($n = 12$) and (d) PBMC of Aza treated MDS patients before treatment ($n = 8$), on day 15 of the first cycle ($n = 8$) and after 3 cycles ($n = 6$). (e) Expression of Tbet ($n = 5$; $p > 0.10$), RoRγT ($n = 4$; $p > 0.10$) and FoxP3 ($n = 5$; $p < 0.08$) transcripts (relative to the HPRT gene) in $CD4^{pos}CD25^{neg}$ cells cultured *in vitro* with Aza. *p*-Values are from 2-sided paired Student's *t*-tests.

cytokines may be related to the outgrowth of (transformed) blasts in high risk MDS cases. Many genes are regulated by epigenetics, among them are genes involved in the regulation of the immune response, such as FoxP3. Previous *in vitro* studies have shown that methylation of the FoxP3 promoter plays an important role in the regulation of FoxP3 expression and demonstrated an up-regulation of FoxP3 expression in $CD25^{neg}CD4^{pos}$ T-cells upon stimulation in the presence of Aza or decitabine [15,18,21]). Here we confirm these results and demonstrate that during T-cell activation Aza increases FoxP3 mRNA and protein expression from both $CD25^{dim}$ and $CD25^{neg}CD4^{pos}$ T-cells, while Aza did not affect proliferation of isolated $CD25^{hi}CD4^{pos}$ T-cells which are highly enriched for natural Treg. FoxP3 expression in humans, particular in cultured T-cells, can also be a sign of T-cell activation rather than a sign of Treg conversion [23]. Indeed, cytokine expressing FoxP3 positive cells were observed both in control as well as Aza cultures as was previously shown by Nagar et al. [21]. However we show here that at least a proportion of the converted FoxP3 did not express IFNγ or TNFα and that Aza cultured $CD25^{hi}$ cells had a stronger capacity to inhibit autologous $CD4^{pos}$ T-cell proliferation as compared to vehicle cultured cells. These data show that during T-cell activation Aza induces conversion of Tconv rather than affecting the proliferation

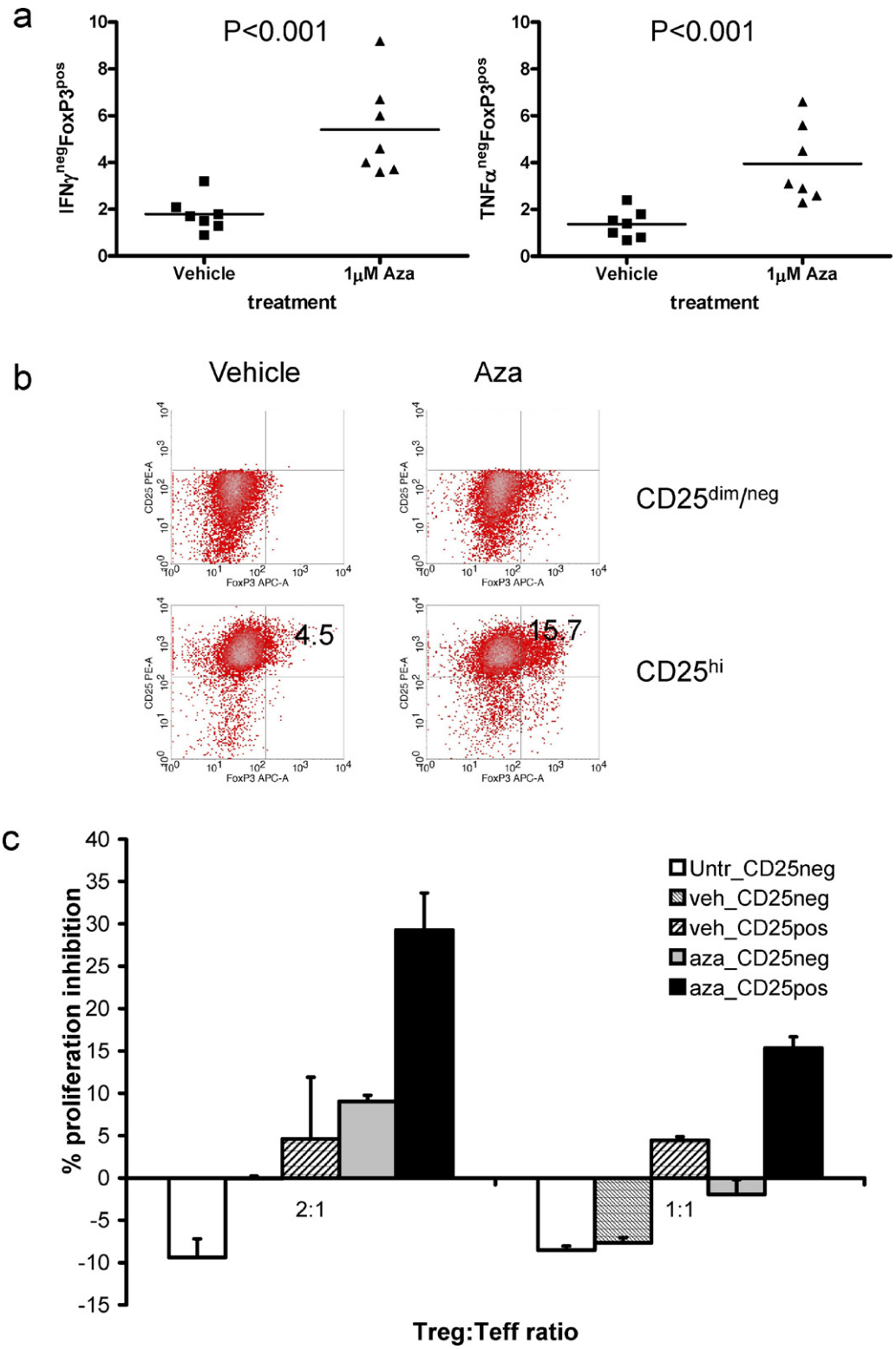

Fig. 5. Analysis of regulatory function of FoxP3^{pos} cells. Proliferation and activation of isolated CD4^{pos} T-cells was induced by CD3 cross-linking. Vehicle or 1 μM of Aza was added at the start of the culture and on two consecutive days of culture, cells were harvested on day 6. (a) Cells were stimulated with PMA/ionomycin in the presence of Brefeldin A and analyzed after 16 h for the presence of IFNγ^{pos}FoxP3^{neg} (left panel) and TNFα^{pos}FoxP3^{neg} (right panel) cells ($n = 7$). p-Values are from 2-sided paired Student's t-tests. (b) FoxP3 analysis of sorted CD25$^{neg/dim}$ (top panels) and CD25^{hi} (bottom panels) cells of CD4^{pos} T-cell cultured in the presence of vehicle (left panels) or Aza (right panels). Numbers in upper right quadrant are the percentage of CD25^{hi}FoxP3^{pos} Treg. (c) Treg assay: untreated CD4^{pos}CD25^{neg} control cells (Untr_CD25neg), vehicle treated CD25$^{neg/dim}$ (veh_CD25neg) and CD25^{hi} (veh_CD25pos) cells and Aza treated CD25$^{neg/dim}$ (aza_CD25neg) and CD25^{hi} (aza_CD25pos) cells were added to autologous CD3 stimulated CD4^{pos} T-cells at a ratio of 2:1 and 1:1 and proliferation was measured by CFSE dilution. The proliferation inhibition by the different Treg populations is plotted.

of natural Treg. To investigate whether Aza also induces an increase in Treg frequencies *in vivo*, we analyzed Treg frequencies in nine high risk MDS patients who were treated with Aza. A trend toward increased Treg was observed on day 15 of the first cycle, but Treg numbers were reduced again to levels before treatment at the end of the third cycle. The lack of a sustained increase in FoxP3 positive cells may be related to the lack in clinical response as only 2 patients had a clinical response. However, only one of these two patients was analyzed after 3 cycles and also did not show a sustained increase in FoxP3 positive cells (Supplementary Fig. 4A). Intrinsic differences between $CD4^{pos}$ T-cells derived from healthy donors and high-risk MDS patients are unlikely to be involved in this effect since $CD4^{pos}$ T-cells isolated from Aza naive high-risk MDS patients stimulated *in vitro* with Aza showed a comparable increase in $FoxP3^{pos}$ Treg as the healthy donor derived $CD4^{pos}$ T-cells (data not shown). The lack of a significant sustained increase in Treg *in vivo* may rather be due to the dose of Aza the T-cells are exposed to *in vivo.* As we demonstrate here, the window of Aza levels at which it induces FoxP3 in $CD4^{pos}$ T-cells is quite narrow; at 0.1 μM the drug has no effect, while at 10 μM the drug is toxic (see Fig. 1b). Furthermore, the *in vitro* increase in FoxP3 expression was observed after a strong stimulus by immobilized anti-CD3 while T-cell activation in the peripheral blood, particularly of high-risk MDS patients, may be less evident. Unfortunately the material obtained was not sufficient to test for functional Treg activity or to analyze the methylation status of the Treg specific demetylated region (TSDR) [26] to confirm that the $CD3^{pos}CD4^{pos}CD127^{lo/neg}$ cells are indeed functional Treg.

In cancer, both positive and negative associations with IL-17 producing cells have been described [27–30]. This may be related to their microenvironment dependent plasticity and capacity to co-produce other inflammatory cytokines such as IFNγ [31,32]. $CD4^{pos}$ T-cells expressing IL-17 with (T_H17-1) or without IFNγ (T_H17), are found in inflamed gut area's of Crohn's disease patients [33] indicating that they contribute to pathogenic chronic inflammation. The relative contribution of T_H17-1 and T_H17 in inflammatory disorders and cancer is still unclear. We have found that the small fraction of T_H17-1 cells is not significantly affected by Aza while we show a significant reduction in T_H17 by Aza both *in vitro* and *in vivo*, whereas the overall frequency of total $CD4^{pos}$ IL-17 producing cells was not significantly affected. These data suggest that the development of these two subsets is differentially regulated. Recent data showing that TGFβ is crucial for the development of $IL\text{-}17^{pos}IFN\gamma^{neg}$ T_H17 while IL-1β is important for the development of $IL\text{-}17^{pos}IFN\gamma^{pos}$ T_H17-1 cells also suggest this [34]. In addition IL-6 plays a role in the differentiation and proliferation of T_H17. As TGFβ and IL-6 are produced by (among other cells) T_H2 cells, these cytokines may indeed be inhibited by Aza [35] as it induces a predominant T_H1 response ([36] and this paper). Larger *in vivo* studies relating T_H17 and T_H17-1 subsets frequencies with clinical responses to (Aza) treatment may shed more light on the relevance of these subsets. Similar to solid tumors, opposing observations regarding T_H17 in MDS have been described. While T_H17 were increased in the peripheral blood [12], T_H17 were dysfunctional and reduced in frequency in the bone marrow of low-risk patients but increased in the bone marrow of high risk patients [13], suggesting a detrimental effect of IL-17 in the tumor microenvironment. Unfortunately we did not analyze CD4 T-cell polarization in the bone marrow of the high risk patients treated with Aza in the present study and whether Aza affects T_H17 in the bone marrow remains to be investigated.

An increased T_H17:Treg ratio in the peripheral blood of low risk MDS patients as compared to high risk MDS patients has been correlated to increased bone marrow apoptosis [12]. We therefore analyzed the effect of Aza treatment on the T_H17:Treg ratio. *In vitro* this ratio is significantly reduced and due to the initial increase in Treg and the decline in IL-17 producing T-cells, the T_H17:Treg ratio is significantly reduced *in vivo* 15 days after the start of treatment. When analyzing the change in Treg and T_H17 frequencies in individual patients no or a relatively modest immune modulation was observed in both patients with stable disease (showing a slight decrease in Treg, rather than an increase) and a more or less stable T_H17:Treg ratio on day 15. On the other hand both responding patients showed comparable immune modulation by Aza treatment to the four progressive patients (Supplementary Fig. 4). Studies with extended patient groups will have to be performed to elucidate whether a lack of immune modulation by Aza is related to better clinical responses.

Within the predominantly naïve $CD25^{neg}$ T-cell pool the proportion of IL-17 producing cells was significantly enhanced in the presence of Aza. In contrast, when the total $CD4^{pos}$ T-cell pool was activated in the presence of Aza the number of T_H17 was significantly reduced, suggesting that Aza facilitated the development of T_H17 from naïve $CD4^{pos}$ T-cells but reduced the differentiation of T_H17 from the memory pool, particularly in the presence of Treg (see Figs. 2a and 3d). It has been shown that Treg can differentiate into T_H17 and *vice versa* [37,38], our results suggest that Aza may facilitate the differentiation of Treg from T_H17, however studies with isolated populations are needed to corroborate this.

Conflicting results regarding the effects of Aza on TNFα and IFNγ production have been reported, Sánchez-Abarca et al. demonstrated a significant decrease in production by anti-CD3 stimulated lymphocytes early (on day 2) after stimulation in the presence of Aza [18], in contrast, Yano et al. have reported a T_H2 to T_H1 conversion under the influence of Aza [16] and Nader et al. demonstrated an increase in TNFα and IFNγ production by Aza. In accordance with the latter studies we observed a significant increase in IFNγ producing cells after *in vitro* stimulation in the presence of Aza of both healthy donor and high-risk MDS derived $CD4^{pos}$ T-cells (not shown). TNFα production was only significantly increased in recently activated T-cells. However, we observed no significant changes in the proportion of TNFα or IFNγ producing cells after Aza treatment *in vivo*. Why the effect on IFNγ and TNFα producing cells is lacking *in vivo* is not clear, but it may also be due to maximum plasma levels, a lack of general T-cell activation or effects of Aza on other cells, *e.g.* antigen presenting cells, which were not taken into account in the *in vitro* studies. Together with our results showing no change in Tbet and RoRγT expression upon Aza treatment, these data suggest that the IL-17 and TNFα promoters are differentially methylated in recently activated and resting T-cells, and that Aza may enhance primary IL-17 responses, while in the case of TNFα, pre-existing responses will predominantly be affected.

In conclusion, the inhibition of T_H17 cells as well as increased numbers of Treg upon Aza treatment *in vitro* and to a somewhat lesser extend *in vivo* indicate a predominant effect of Aza on the T_H17-Treg axis. In this small group no relation with this effect to clinical response was apparent and larger studies are needed to establish a relation with clinical responses. It is clear however that next to its cytotoxic effects, Aza has clear immune modulatory effects which should be taken into account in clinical practice.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

This work was supported by Avanti-STR (JM Ruben). Avanti-STR did not have a role in study design; in collection, analysis, and interpretation of data; in the writing of the report; nor in the decision to submit the paper for publication.

Contributions. Authors contributed to the following: conception and design of the study [HJB, GJO, AAL], acquisition of the data,

analysis and interpretation of the data [HJB, JRM, AC] drafting the manuscript [HJB], critical revision of the manuscript [AC, TMW, AAL], final approval of the version to be submitted [all authors].

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.leukres.2012.03.026.

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